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Abstract: Experimental autoimmune myocarditis (EAM) represents a CD4(+) T helper (Th) cell-mediated mouse model of inflammatory heart disease. Interferon (IFN)- , typically produced by Th1 cells, reduces EAM severity in myosin heavy-chain-(MyHC)- peptide/Complete Freund adjuvant-immunized mice. Thus, developing a vaccination strategy that promotes differentiation of Th1 cells may be beneficial in EAM. FMS-like tyrosine kinase 3 ligand (Flt3L)-induced splenic CD8 (+) dendritic cells (DC), which produce interleukin (IL)-12p35, were identified to selectively induce biased differentiation towards Th1. Mice vaccinated with MyHC- -loaded Flt3L-induced splenic CD8 (+) DC were protected from EAM. In contrast, when Flt3L-induced splenic CD8 (+) DC were pre-stimulated and over-activated with LPS and CD40 antibodies or loaded with unspecific OVA(323-339) peptide instead of MyHC- peptide, mice developed similar disease scores as non-vaccinated controls. Vaccination efficacy depended on IFN- , since CD8 (+)-vaccinated IFN- R(-/-) mice were not protected. Importantly, splenic CD8 (+) vaccination was independent of regulatory T cells. Taken together, Flt3L-induced dendritic cell-based antigen-specific vaccination limits expansion of auto-reactive Th cells and protects mice from autoimmune heart inflammation.

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Vaccination with Flt3L-induced CD8α$^+$ dendritic cells prevents CD4$^+$ T helper cell-mediated experimental autoimmune myocarditis

Alan Valaperti[a,b*], Mototsugu Nishii[b,c], Davide Germano[a,d], Peter P Liu[b], and Urs Eriksson[a,e]

[a]Division of Cardioimmunology, Cardiovascular Research, Institute of Physiology and Center for Integrative Human Physiology, University of Zurich, Switzerland

[b]Division of Cardiology, Heart and Stroke/Richard Lewar Centre of Excellence, Toronto General Research Institute, University Health Network, University of Toronto, Ontario, Canada

[c]Department of Internal Medicine and Cardiology, Kitasato University School of Medicine, Japan

[d]Preclinical Safety, Discovery and Investigative Safety, Novartis Pharma AG, Basel, Switzerland

[e]Department of Medicine, GZO - Zurich Regional Health Center, Wetzikon, Switzerland

Running title: Vaccination against autoimmune myocarditis

[*] Corresponding address: Alan Valaperti, Ph.D.
Division of Cardioimmunology, Cardiovascular Research
Institute of Physiology and Center for Integrative Human Physiology
University of Zurich
Winterthurerstrasse 190
CH-8057 Zurich, Switzerland
Phone: +41-76-512-7343
Email: alan.valaperti@me.com

Key words: dendritic cells; Th1; Th17; autoimmunity; Flt3L; myocarditis.

Abbreviations: EAM, experimental autoimmune myocarditis; Th, CD4$^+$ T helper; Flt3L, FMS-like tyrosine kinase 3 Ligand; MyHC, myosin heavy-chain.

Abstract

Experimental autoimmune myocarditis (EAM) represents a $CD4^+$ T helper (Th) cell-mediated mouse model of inflammatory heart disease. Interferon (IFN)-γ, typically produced by Th1 cells, reduces EAM severity in myosin heavy-chain-(MyHC)-α peptide/Complete Freund adjuvant-immunized mice. Thus, developing a vaccination strategy that promotes differentiation of Th1 cells may be beneficial in EAM.

FMS-like tyrosine kinase 3 Ligand (Flt3L)-induced splenic $CD8\alpha^+$ dendritic cells (DC), which produce interleukin (IL)-12p35, were identified to selectively induce biased differentiation towards Th1. Mice vaccinated with MyHC-α-loaded Flt3L-induced splenic $CD8\alpha^+$ DC were protected from EAM. In contrast, when Flt3L-induced splenic $CD8\alpha^+$ DC were pre-stimulated and over-activated with LPS and αCD40 antibodies or loaded with unspecific $OVA^{323-339}$ peptide instead of MyHC-α peptide, mice developed similar disease scores as non-vaccinated controls. Vaccination efficacy depended on IFN-γ, since $CD8\alpha^+$-vaccinated IFN-$\gamma R^{-/-}$ mice were not protected. Importantly, splenic $CD8\alpha^+$ vaccination was independent of regulatory T cells.

Taken together, Flt3L-induced dendritic cell-based antigen-specific vaccination limits expansion of auto-reactive Th cells and protects mice from autoimmune heart inflammation.

1. Introduction

Dilated cardiomyopathy (DCM) represents the most common cause of heart failure in young patients and often evolves from viral myocarditis [1-3]. Clinical observations suggest that post-infectious autoimmunity promotes disease development [4, 5]. Experimental autoimmune myocarditis (EAM) is a mouse model for $CD4^+$ Th cell-mediated post-infectious myocarditis [6-9] and can be induced in susceptible mouse strains by immunization with myosin heavy chain (MyHC)-α peptide together with Complete Freund's adjuvant (CFA) [10, 11]. In wild-type mice, disease severity peaks between 20 and 23 days after immunization.

In the past years, studies have demonstrated that IL-17A accounts for disease development [6-8], while IFN-γ was considered as a protective cytokine in MyHC-α peptide/CFA-induced EAM and viral myocarditis [6, 12-17]. Indeed, IFN-γ ligand and IFN-γ receptor deficient mice were more susceptible to EAM than their wild-type counterparts [14, 15]. Furthermore, mice lacking of the typical Th1 cell T-box transcription factor Tbet showed increased amounts of IL-17A and consequently higher myocarditis severity than wild-type mice [6]. Consistently, neutralization of IL-17A by active vaccination or by serial injections of anti-IL-17A antibodies significantly reduced EAM, but did not confer complete protection [7, 8]. In fact, recent reports showed that IL-17A deficiency *per se* does not determine myocarditis severity, but rather protects from development of an inflammatory DCM phenotype [9, 18]. Nevertheless, IL-17A promotes recruitment of $CD11b^+$ monocytes into the heart of wild-type mice before the peak of EAM [8, 9]. Other studies suggest that lack of IL-17A can counter-balance and therefore increase the production of protective IFN-γ. [19-21].

On one hand, dendritic cells are potent antigen-presenting cells of the innate immune system that induce primary T cell responses [22-26] and inflammatory cytokines release [27, 28]. After stimulation with LPS plus αCD40 antibodies, GM-CSF-induced peptide-pulsed bone marrow-derived dendritic cells (BMDC) ($CD8\alpha^-CD11c^+CD11b^+$), produce IL-6 and TGF-β

and induce auto-aggressive $CD4^+$ Th17 cells and autoimmune diseases, such as experimental autoimmune myocarditis (EAM) [11, 26, 29, 30] and experimental autoimmune uveitis (EAU) [31]. On the other hand, several subsets of regulatory DC have been described so far. A specific monocytes-derived subset of nitric oxide- and TNF-α-releasing DC subset, for example, confines auto-reactive T cell expansion in EAM [32]. Moreover, several lines of evidence indicate that splenic DC subpopulations of lymphoid origin also modulate T cell differentiation and promote various cytokine production [33-35]. Indeed, Flt3L-induced splenic $CD8\alpha^+CD11c^+CD11b^-$ DC have been shown to produce IL-12 and promote differentiation and expansion of $CD4^+$ Th1 cells [33-36].

Given the protective role of IFN-γ in EAM, we hypothesized that vaccination with Flt3L-induced self-antigen-loaded splenic $CD8\alpha^+$ DC specifically expands heart-specific $CD4^+$ Th1 cells, which may protect from cardiac auto-immunity after MyHC-α peptide/CFA-induced EAM.

2. Material and methods

2.1. Mice

DO11.10, IFN-$\gamma R^{-/-}$, and CD45.1 mice were previously described [8, 15]. Six- to eight-weeks-old BALB/c male mice were used. All mice were housed in an optimized hygienic area. Animal experiments were conducted in accordance with the Swiss federal laws and institutional guidelines.

2.2. Immunization protocol

Mice were immunized with 150 µg of the MyHC-α (MyHC-$\alpha_{614-634}$) peptide Ac-SLKLMATLFSTYASAD-OH (Caslo, Denmark) emulsified 1:1 in PBS/CFA (1 mg/ml, H37Ra; Difco) as described [11].

2.3. Histopathology and immunohistochemistry

Myocarditis severity was graded blindly and independently on H&E-stained sections by two investigators, using grades from 0 to 4 as previously described [26].

2.4. Isolation of Flt3L-induced splenic CD8α$^+$ dendritic cells

Wild-type mice were daily intra-peritoneally treated for ten days with 10 µg/mouse of human recombinant FMS-like tyrosine kinase 3 Ligand (Flt3L). After Flt3L treatment, spleens were digested with Collagenase type IV (Worthington, Lakewood, NJ) and red blood cells were lysed with ACK. Cell suspensions were stained with the CD8α$^+$ Dendritic Cell Isolation Kit (Mouse, MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the provider's instructions. Stained cells were sorted using an autoMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany), following the provider's protocol. CD8α$^+$ DC were seeded at a density of $1.5x10^6$ cells/ml, pulsed with 2 µg/ml MyHC-α-peptide only or pulsed and/or stimulated with 0.1 µg/ml lipopolysaccharide (LPS) plus 5 µg/ml αCD40 antibodies for 4 hours. After washing, DC were re-suspended in PBS at a density of $2.5x10^6$/ml. Each

vaccinated syngenic mouse received 0.5 Mio CD8α^+ DC by i.v. injection three times, every second day, as described in Fig. 3A.

2.5. FACS analysis

Cell suspensions were stained using fluorochrome-conjugated mouse-specific antibodies as described in detail in the Supplementary data.

2.6. T cell proliferation assays and ELISA

In vitro co-cultivation of sorted CD4$^+$ T cells (magnetic beads, Miltenyi Biotec, Bergisch Gladbach, Germany) with CD8α^+ DC is described in the Supplementary data. Cytokines were measured using commercially available ELISA kits (R&D Systems).

2.7. In vivo CD4$^+$ T cell migration and proliferation

CD4$^+$ T cells (DO11.10 TCR) were first labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) and then injected intravenously at 1x10^7 cells per mouse. Twenty-four hours later, each mouse was i.v. injected with 0.5 Mio of OVA$^{323-339}$-loaded CD8α^+ DC previously stimulated *in vitro* with LPS (0.1 μg/ml) plus αCD40 antibodies (0.5 μg/ml) for 4 h, or i.v. injected with 0.5 Mio of untreated OVA$^{323-339}$-loaded CD8α^+ DC. Forty-eight hours later, axillary lymph nodes (ALN), inguinal lymph nodes (ILN), mediastinal lymph nodes (MLN), and spleens were collected. Lymph nodes and spleens were digested with Collagenase type IV and then homogenized prior to cell staining.

2.8. Statistics

The Mann-Whitney *U* test was used for the evaluation of severity scores. Normally distributed data, such as proliferation responses and cytokine levels, were compared using unpaired, two-tailed Student's *t*-test. Statistical analysis was conducted using Prism 4 software (GraphPad Software). Differences were considered as statistically significant for $*p < 0.05$.

3. Results

3.1. Phenotype of Flt3L-induced splenic CD8α$^+$ dendritic cells

After showing that CD4$^+$ Th1 cells conferred protection from EAM [8], we used DC expressing CD8α$^+$ to promote specifically differentiation of IFN-γ-producing CD4$^+$ Th1 cells [35, 36]. Induction and maturation of CD8α$^+$ DC was performed *in vivo* by serial injections of Flt3L in wild-type mice for ten days. CD8α$^+$ DC were sorted from other splenic dendritic cell populations and analysed by flow cytometry. As shown in Fig. 1A, splenic DC highly expressed CD8α and CD11c, but low CD11b, indicating their lymphoid origin. Sorted Flt3L-induced CD8α$^+$ DC were left untreated (hereafter referred as to CD8α$^+$) or stimulated *in vitro* for 4 h with LPS plus αCD40 antibodies (hereafter referred as to LPS-αCD40/CD8α$^+$) or with TNF-α (hereafter referred as to TNF/CD8α$^+$). Surface marker phenotype of LPS-αCD40/CD8α$^+$ and TNF/CD8α$^+$ DC displayed elevated levels of the co-stimulatory molecules CD40, CD80 and CD205, while CD86 and MHC II expression was similar in all groups (Fig. 1B and Suppl. Fig. S1A). Cell-free supernatants were analysed for cytokines using ELISA. LPS-αCD40/CD8α$^+$ DC produced significantly higher levels of IL-1β and IL-6 than CD8α$^+$ DC, while TGF-β and IL-23 production was comparable in both groups (Fig. 1C). Flt3-induced CD8α$^+$ DC were also compared to untreated GM-CSF-induced BMDC, which produced higher amounts of IL-6, but lower IL-12p35, after analysis by flow cytometry (Suppl. Figs. S1B-S1C).

CD4$^+$ Th cell differentiation has been shown to be also determined by the interaction between delta-like receptors on DC and Notch ligands on T cells [37, 38]. We observed that CD8α$^+$ DC expressed high RNA levels of delta-like 1, which promotes CD4$^+$ Th1 cell differentiation [37], while LPS-αCD40/CD8α$^+$ DC expressed high levels of delta-like 4, which supports development of CD4$^+$ Th17 cells [38] (Fig. 1D). These data suggest that CD8α$^+$ DC may specifically promote differentiation of CD4$^+$ Th cell subsets.

165

166 *3.2. CD8α$^+$ and LPS-αCD40/CD8α$^+$ DC have the same potential to prime T cells, but polarise*
167 *different CD4$^+$ Th cell subsets*

168 To investigate the potential of CD8α$^+$ DC to prime naïve T cells *in vitro*, we co-
169 cultivated OVA$^{323-339}$-peptide-pulsed CD8α$^+$ and LPS-αCD40/CD8α$^+$ DC with titrating
170 amounts of OVA-specific CD4$^+$ T cells. T cell proliferation assessed by ^{3}H-thymidine
171 incorporation showed that CD8α$^+$ and LPS-αCD40/CD8α$^+$ DC induced similar proliferation
172 after 48 and 72 hours of incubation (Fig. 2A). Differentiation of CD4$^+$ Th1 cells is dependent
173 on Tbet, while the CD4$^+$ Th17 lineage depends on the transcription factor retinoic-acid-
174 receptor-related orphan receptor (ROR)γt [6, 21, 39]. *In vitro*, CD8α$^+$ DC showed higher Tbet
175 RNA, but lower RORγt RNA expression, than LPS-αCD40/CD8α$^+$ DC (Fig. 2B-2C).
176 Consistently, CD8α$^+$ DC produced higher amounts of IFN-γ, but lower amounts of IL-17A,
177 than LPS-αCD40/CD8α$^+$ DC (Fig. 2B-2C).

178 To study the priming capacity of CD8α$^+$ DC *in vivo*, CD45.2 CD8α$^+$ or LPS-
179 αCD40/CD8α$^+$ DC pulsed with OVA$^{323-339}$-peptide were intravenously injected into CD45.1
180 wild-type recipient mice 24 hours before transfer of CD45.2 CFSE-labelled DO11.10 (OVA-
181 specific) CD4$^+$ T cells. Migrating CD8α$^+$ and LPS-αCD40/CD8α$^+$ DC, which exerted their
182 proliferative potential exclusively in mediastinal LN and spleen, showed comparable capacity
183 to prime CD4$^+$ T cell (Fig. 2D). However, CD8α$^+$ DC promoted proliferation of IFN-γ-
184 producing DO11.10 CD4$^+$ T cells, while LPS-αCD40/CD8α$^+$ DC induced both IFN-γ- and IL-
185 17A-producing DO11.10 CD4$^+$ T cells (Fig. 2D). Taken together, CD8α$^+$ DC support
186 differentiation of antigen-specific IFN-γ-producing CD4$^+$ Th1 cells, while LPS-CD40/CD8α$^+$
187 DC support polarisation of both CD4$^+$ Th1 and IL-17A-producing CD4$^+$ Th17 cells.
188

3.3. Vaccination with MyHC-α-loaded Flt3L-induced CD8α$^+$ DC prevents EAM

EAM is inducible in BALB/c and A/J mice with a dominant CD4$^+$ Th2 phenotype, while CD4$^+$ Th1-dominant C57Bl/6 mice are largely resistant [40, 41]. We therefore tested the potential of CD8α$^+$ DC to promote *in vivo* a Th1 response in the Th2 dominant BALB/c background. CD8α$^+$ and LPS-αCD40/CD8α$^+$ DC were pulsed with MyHC-α-peptide for 4 hours. Mice were vaccinated with three intravenous injections of DC at days -7, -5, and -3 before induction of myocarditis with MyHC-α-peptides emulsified in CFA at day 0 and day 7 (Fig. 3A). Negative controls for vaccination were vehicle (PBS) control and vaccination with three intravenous injections of untreated CD8α$^+$ pulsed with OVA$^{323-339}$-peptide. As hypothesized, vaccination with CD8α$^+$ DC protected mice from EAM (Fig. 3B). In contrast, PBS control and OVA$^{323-339}$-pulsed CD8α$^+$-vaccinated control mice exhibited severe myocarditis at day 21, while mice vaccinated with LPS-αCD40/CD8α$^+$ DC had slightly decreased EAM incidence (Fig. 3B). Myocarditis scores were irrelevant from day 0 to day 21 in mice vaccinated with CD8α$^+$ DC, suggesting that vaccination with CD8α$^+$ DC, in addition to prevent EAM, was not pathogenic (Figs. 3C-3D). On the other side, vaccination with LPS-αCD40/CD8α$^+$ DC induced mild myocarditis at day 0 and exerted irrelevant protection against EAM at day 21 when compared to PBS control or OVA$^{323-339}$-pulsed CD8α$^+$-vaccinated control mice (Figs. 3C-3D). In addition, treatment with three intravenous injections of GM-CSF-induced BMDC pulsed with MyHC-α-peptide showed even more aggravated disease from day 0 to day 21 (Fig. 3E). Taken together, these data indicate that only antigen-specific vaccination with MyHC-α-pulsed CD8α$^+$ DC protects from EAM.

3.4. Antigen-specific IFN-γ-producing CD4$^+$ Th1 confer protection from EAM

212 $CD4^+$ Th cell differentiation occurs few days after myocarditis induction. At day 2,
213 sorted splenic $CD4^+$ T cells proliferated to similar extent in all analysed groups, while at day 9,
214 vaccination with $CD8\alpha^+$ DC inhibited $CD4^+$ T cell proliferation when compared to LPS-
215 αCD40/$CD8\alpha^+$ DC or PBS control vaccination (Fig. 4A). IFN-γ production was observed in
216 both DC-vaccinated mice, but $CD8\alpha^+$ DC vaccination induced higher levels of IFN-γ than
217 LPS-αCD40/$CD8\alpha^+$ DC or PBS vaccination. On the other side, IL-17A production was lower
218 in $CD8\alpha^+$ DC-vaccinated mice than LPS-αCD40/$CD8\alpha^+$ DC-vaccinated mice or PBS only
219 controls (Fig. 4B). Taken together, $CD8\alpha^+$ DC vaccination promotes protective $CD4^+$ Th1
220 cells, while LPS-αCD40/$CD8\alpha^+$ DC vaccination induces a mixed $CD4^+$ Th1 and $CD4^+$ Th17
221 response, which was not protective.
222
223 *3.5. Protection conferred by Flt3L-induced $CD8\alpha^+$ DC depends on IFN-γ*
224 Mice lacking of IFN-γ or IFN-γ receptor (IFN-γR) develop severe EAM scores after
225 MyHC-α/CFA immunization [13-15], while IFN-γ is critical for a negative feedback loop
226 confining auto-reactive T cell expansion at the peak of disease [8]. To analyse the role of IFN-γ
227 in our vaccination strategy, we injected wild-type (IFN-$\gamma R^{+/+}$) and IFN-$\gamma R^{-/-}$ mice with wild-
228 type $CD8\alpha^+$ DC as described in Fig. 3A. Vehicle (PBS) was used as negative control for
229 vaccination. Myocarditis severity scores at day 21 indicated that $CD8\alpha^+$ DC vaccination was
230 efficient in wild-type mice only, but not in IFN-$\gamma R^{-/-}$ mice, while PBS control mice showed
231 typical EAM development for wild-type and IFN-$\gamma R^{-/-}$ mice [14, 15] (Fig. 5A-5B). Importantly,
232 $CD8\alpha^+$ DC vaccination in wild-type, but not in IFN-$\gamma R^{-/-}$ mice, significantly reduced MyHC-α-
233 specific $CD4^+$ T cell proliferation (Fig. 5C). These data suggest that IFN-γ is the major
234 regulator in the protective mechanism conferred by $CD8\alpha^+$ DC vaccination.
235

3.6. GITR$^+$ regulatory T cells are superfluous in the CD8α$^+$ DC vaccination machinery

Beside CD4$^+$ Th1 cells, regulatory T cells (Tregs) can also produce IFN-γ [42]. Looking at the typical Tregs differentiation transcription factor FoxP3, we observed that OVA$^{323-339}$-pulsed CD8α$^+$ and LPS-αCD40/CD8α$^+$ DC co-cultivated *in vitro* with OVA-specific CD4$^+$ T cells induced equal expression of FoxP3 RNA (Fig. 6A). *In vivo*, CD4$^+$FoxP3$^+$GITR$^+$ Tregs were found in equal amounts in the spleens of CD8α$^+$ DC-, LPS-αCD40/CD8α$^+$ DC-, and PBS control-vaccinated mice (Fig. 6B). Similarly, IFN-γ produced in CD4$^+$FoxP3$^+$GITR$^+$ Tregs was comparable in all groups (Fig. 6B).

To test the inhibitory role of Tregs in EAM [43], titrating amounts of GITR$^+$ Tregs sorted from CD8α$^+$ DC-, LPS-αCD40/CD8α$^+$ DC-, or PBS control-vaccinated mice were co-cultivated *in vitro* with splenocytes collected from mice with EAM at day 21. As shown in Fig. 6C, GITR$^+$ Tregs sorted from each group were equally efficient in inhibiting proliferation of MyHC-α-specific splenocytes. These findings indicate that Tregs are not involved in the protective mechanism induced by CD8α$^+$ DC vaccination.

4. Discussion

In this study we propose for the first time a CD8α$^+$ dendritic cell-based vaccination strategy against MyHC-α/CFA-induced EAM. We managed to specifically expand a protective MyHC-α-specific CD4$^+$ Th1 response in otherwise Th2-biased BALB/c mice. This protective CD4$^+$ Th1 response was IFN-γ dependent because our vaccination strategy was not effective in IFN-γ receptor deficient mice. Importantly, the DC vaccination protocol worked with MyHC-α-loaded non-stimulated Flt3L-inudced CD8α$^+$ DC only, which induced a selective CD4$^+$ Th1 cell response. In contrast, LPS- and αCD40-stimulated Flt3L-induced CD8α$^+$ DC triggered expansion of mixed Th cell subsets and were not protective at all.

CD4$^+$ Th cell differentiation has been thoroughly studied in the past ten years. From the first concept of the Th1/Th2 paradigm, new results indicated the presence of another type of effector Th cells, namely Th17 cells, that produced IL-17A and exhibited effector functions [44]. Th17 cells are supposed to be potent inducers of tissue inflammation and are associated with various autoimmune diseases. There is no doubt that IL-17A promotes chronic inflammation and heart failure in the EAM model, but the specific role of Th17 cells for induction and early expansion of heart-specific T cell responses is still unclear [9, 18]. In this study, however, we found that only a selective CD4$^+$ Th1 cell response provides a protective memory against subsequent MyHC-α/CFA immunization. Accordingly, expansion of a mixed population of antigen-specific Th1 and Th17 cells observed in LPS-αCD40/CD8α$^+$ DC-vaccinated mice, however, was not protective. We can also not exclude a role for other pro-inflammatory cytokines, such as GM-CSF, in such mixed Th response [45]. Future studies specifically focused on EAM will be necessary to figure out which pattern of cytokines produced by Th cells is responsible for EAM induction.

We have previously demonstrated the importance of IFN-γ in the negative feedback mechanism that confines auto-reactive T cell expansion in EAM [8]. IFN-γ, indeed, initiates the

final maturation step of heart-infiltrating monocytes to a nitric oxide-releasing anti-inflammatory DC subset, which suppresses activated auto-reactive Th cells [32]. In the present study, increased IFN-γ production reflected suppressed early T cell expansion and reduced IL-17A levels after vaccination with protective $CD8\alpha^+$ DC. Thus, IFN-γ exerts its protective effects through different mechanisms at different time-points during the process of early T cell priming, expansion and disease progression [6, 13, 15]. We believe that rapid *in vivo* differentiation towards Th1 cells quickly suppresses pathogenic Th17 cells, which is critical for disease progression in EAM. In fact, counter-regulation between Th1 and Th17 cells has been widely described *in vitro* [19-21, 46]. Nevertheless, here we provide for the first time *in vivo* evidence that $CD8\alpha^+$ DC-promoted Th1 differentiation negatively regulates autoimmune responses.

IFN-γ production is not a prerogative of T cells only. Indeed, DC and Treg also produce relevant amounts of IFN-γ [42, 47, 48]. However, since IFN-γ production was almost undetectable in $CD8\alpha^+$ DC, we investigated if regulatory T cells, which produce IFN-γ under particular stimulatory signals to inhibit Th cells [42], were involved in the induction of protective IFN-γ. Sorting GITR-expressing Tregs after vaccination [43], however, showed that the inhibitory potential of Tregs was not affected by our vaccination strategy. All together, the non-significant production of IFN-γ by $CD8\alpha^+$ DC and Tregs supports our hypothesis that $CD4^+$ Th1 is indeed the cell population that produces IFN-γ and protects from EAM.

Together with $CD8\alpha^+$ DC, Flt3L also induces proliferation of plasmacytoid DC (pDC) [34]. We decided to use $CD8\alpha^+$ DC because of their bigger potential to stimulate $CD4^+$ T cells compared to the very weak stimulatory potential of pDC [49]. Although Flt3L has been described to worsen autoimmune diseases due to over-expression of stimulatory signals from pDC, our vaccination strategy starts from serial injections of Flt3L to promote $CD8\alpha^+$ DC *in*

300 *vivo*, which are then sorted and injected into syngenic mice, facilitating the development of
301 autologous dendritic cell-based vaccination strategies [50].

302 Given the evidence that IL-17A plays a critical role in myocarditis progression and
303 heart failure development rather than during early auto-reactive T cell priming [9], our
304 vaccination strategy may be of considerable clinical interest. If it is feasible to selectively bias a
305 Th1 response in patients during early myocarditis, it may be possible to quench the IL-17A-
306 dependent deleterious late process which leads to end-stage inflammatory cardiomyopathy.
307

5. Conclusions

Here we described for the first time an Flt3L-induced CD8α^+ DC-based antigen-specific vaccination strategy which limits auto-reactive T cell expansion and protects mice from EAM. Moreover, this work contributes to our growing understanding of the role of IFN-γ in inflammatory heart disease. Our data may be a first step towards a novel *ex vivo* DC-based vaccination strategy against inflammatory cardiomyopathy.

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The authors declare no conflict of interest.

Appendix A. Supplementary data

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464 **Figure legends**

465 **Fig. 1.** Phenotype of Flt3L-induced splenic CD8α$^+$ dendritic cells.

466 (A) Purity of sorted splenic dendritic cells checked by analysing expression of the surface
467 markers CD8α$^+$, CD11c, and CD11b by flow cytometry.

468 (B) Expression of surface co-stimulatory markers MHCII, CD40, CD80, CD86, and CD205 on
469 CD8α$^+$ or LPS-αCD40/CD8α$^+$ DC analysed by flow cytometry after 4 h stimulation.
470 Histograms were gated on CD8α$^+$/CD11c$^+$ DC. The grey area represents isotype control. Mean
471 fluorescence intensity is shown.

472 (C) Production of IL-1β, IL-6, IL-23, and TGF-β in CD8α$^+$ (black bars) and LPS-
473 αCD40/CD8α$^+$ (white bars) DC in cell-free supernatants collected after 4 h stimulation
474 measured by ELISA.

475 (D) RNA expression of delta-like 4 and delta-like 1 in CD8α$^+$ (black bars) and LPS-
476 αCD40/CD8α$^+$ (white bars) DC after 4 h stimulation measured by qRT-PCR. GAPDH was
477 used as internal control.

478 Means ± s.d. Values measured from one out of three independent experiments (A-D) performed
479 in triplicates (C-D) are shown. *$p < 0.05$.

480

481 **Fig. 2.** CD8α$^+$ DC polarise CD4$^+$ Th1 but not Th17 cells.

482 (A) OVA$^{323-339}$-pulsed CD8α$^+$ or LPS-αCD40/CD8α$^+$ DC co-cultivated with titrating amounts
483 of sorted DO11.10 (OVA-specific) CD4$^+$ T cells. Co-cultures were incubated for 48 h and 72 h.
484 Proliferation of CD4$^+$ T cells was assessed by ^{3}H-thymidine incorporation.

485 (B) 10^5 DO11.10 CD4$^+$ T cells co-cultivated with 10^4 OVA$^{323-339}$-pulsed CD8α$^+$ or LPS-
486 αCD40/CD8α$^+$ DC for 48 h. Relative RNA expression of Tbet, IFN-γ, RORγt, and IL-17A with
487 GAPDH used as internal control is shown.

(C) IFN-γ and IL-17A were measured in cell-free supernatants collected from co-cultures described in (B) by ELISA.

(D) CD45.2 $OVA^{323-339}$-pulsed $CD8\alpha^+$ and LPS-αCD40/$CD8\alpha^+$ DC intravenously injected into CD45.1 wild-type mice after injection of CFSE-labelled OVA-transgenic $CD4^+$ T cells (CD45.2). CFSE dilutions corresponding to proliferating DO11.10 transgenic $CD4^+$ T cells (detected with the KJ1-26 antibody) were measured in axillary lymph nodes (ALN), inguinal lymph nodes (ILN), mediastinal lymph nodes (MLN) and spleens. All graphs are gated on CD45.2 positive cells. The right graphs illustrate intracellular FACS analysis for IFN-γ and IL-17A in proliferating OVA-transgenic $CD4^+$ T cells. The percentage of peptide-specific IFN-γ and IL-17A production refers to proliferating $CD45.2^+KJ1\text{-}26^+$/CFSE cells.

Means ± s.d. Values measured from one out of three independent experiments performed in triplicates (A-C), or one out of two representative experiments (D) are shown. $*p < 0.05$.

Fig. 3. Vaccination with $CD8\alpha^+$ DC prevents EAM.

(A) Graphic representation of the strategy used to vaccinate mice with $CD8\alpha^+$ DC before myocarditis induction.

(B) Myocarditis score disease at day 21 evaluated according to the criteria described in the Methods section. Each symbol represents disease severity of individual mice, bars are groups means. $**p = 0.0086$ for $CD8\alpha^+$ DC vs. LPS-αCD40/$CD8\alpha^+$ DC, $**p = 0.0045$ for $CD8\alpha^+$ DC vs. PBS control, $**p = 0.0045$ for $CD8\alpha^+$ DC vs. $OVA^{323-339}$-loaded $CD8\alpha^+$ DC.

(C) Time course of EAM in vaccinated mice. Vaccination with $CD8\alpha^+$ DC, LPS-αCD40/$CD8\alpha^+$ DC, PBS control, or $OVA^{323-339}$-loaded $CD8\alpha^+$ DC was performed before induction of myocarditis. Each time-point represents the mean score disease of four mice.

(D) Haematoxylin and eosin staining of heart cross-sections collected at day 21. 20X and 200X magnifications are shown.

(E) Time course of EAM in mice injected with GM-CSF-induced BMDC before induction of myocarditis. Each time-point represents the mean score disease of four mice.

Fig. 4. $CD8\alpha^+$ DC exclusively polarise $CD4^+$ Th1 cells, while LPS-αCD40/$CD8\alpha^+$ DC induce both $CD4^+$ Th1 and $CD4^+$ Th17 cells.

(A) Splenic $CD4^+$ T cells sorted at the indicated days from $CD8\alpha^+$ DC-, LPS-αCD40/$CD8\alpha^+$ DC-, or PBS control-vaccinated mice were co-cultivated on irradiated (25 Gy) MyHC-α-pulsed or un-pulsed $CD4^-$ wild-type splenocytes for 48 h. Peptide specific proliferation was assessed calculating the difference between peptide-pulsed $CD4^+$ T cells and un-pulsed $CD4^+$ T cells (Δ cpm). *$p < 0.05$ for $CD8\alpha^+$ DC vs. LPS-αCD40/$CD8\alpha^+$ DC and for $CD8\alpha^+$ DC vs. PBS control.

(B) IFN-γ and IL-17A were measured after 48 h in the supernatants of the same co-cultures described in (A). IFN-γ and IL-17A concentrations quantified by ELISA are represented as the difference between cytokines produced by MyHC-α-pulsed $CD4^+$ T cells and un-pulsed $CD4^+$ T cells. *$p < 0.05$.

Means ± s.d. Values from 5 culture wells of one out of three representative experiments (A-B) are shown.

Fig. 5. IFN-γ regulates the protective mechanism conferred by $CD8\alpha^+$ DC vaccination.

(A) Myocarditis was induced in wild-type and IFN-$\gamma R^{-/-}$ mice vaccinated with wild-type $CD8\alpha^+$ DC or PBS control as shown in Fig. 3A. Haematoxylin and eosin staining of heart cross-sections collected at day 21 are shown at magnifications of 20X and 200X.

(B) Myocarditis score disease at day 21 evaluated according to the criteria described in the Methods section. Each symbol represents disease severity of individual mice, bars are groups means. *$p = 0.0109$ for wild-type mice vaccinated with $CD8\alpha^+$ vs. wild-type mice with PBS

538 control vaccination, ***p = 0.0002 for wild-type mice vaccinated with CD8α^+ vs. IFN-$\gamma R^{-/-}$
539 mice vaccinated with CD8α^+, n.s. (not significant) p = 0.8808 for IFN-$\gamma R^{-/-}$ mice vaccinated
540 with CD8α^+ vs. IFN-$\gamma R^{-/-}$ mice with PBS control vaccination.
541 (C) Splenic CD4$^+$ T cells sorted from the same vaccinated mice described in (A) were re-
542 stimulated for 48 h on irradiated MyHC-α-pulsed or un-pulsed antigen presenting cells in the
543 presence of 2 µg/ml MyHC-α-peptide. Splenocytes proliferation was assessed by ^{3}H-thymidine
544 incorporation in triplicates. The difference between peptide-specific and peptide-unspecific
545 proliferating cells (Δ cpm) is indicated. Mean ± s.d. values of Δ cpm from 5 individual mice are
546 shown. *$p < 0.05$.
547
548 **Fig. 6.** CD4$^+$FoxP3$^+$GITR$^+$ regulatory T cells are not involved in vaccination protection.
549 (A) 10^5 DO11.10 CD4$^+$ T cells co-cultivated with 10^4 OVA$^{323-339}$-pulsed CD8α^+ or LPS-
550 αCD40/CD8α^+ DC for 48 h. Relative RNA expression of FoxP3 with GAPDH used as internal
551 control is shown.
552 (B) Regulatory T cell analysis after vaccination with CD8α^+ DC, LPS-αCD40/CD8α^+ DC, or
553 PBS control at day 0. Splenocytes were stained for surface markers with CD4, GITR, and for
554 intracellular markers with FoxP3. Tregs depicted on the left graphs are gated on CD4$^+$ cells,
555 while IFN-γ production was assessed gating on CD4$^+$FoxP3$^+$GITR$^+$ cells.
556 (C) Titrating amounts of GITR$^+$ regulatory T cells sorted from mice vaccinated with CD8α^+
557 DC (black circles), LPS-αCD40/CD8α^+ DC (white diamonds), or PBS control (silver triangles)
558 were *in vitro* co-cultivated for 48 h with MyHC-α-restimulated splenocytes sorted from mice
559 with myocarditis in the presence of 2 µg/ml MyHC-α-peptide. Proliferating splenocytes control
560 (grey squares) were cultivated in the absence of Tregs. Splenocytes proliferation was assessed
561 by ^{3}H-thymidine incorporation in triplicates.

562 One out of three representative experiments (A-C) is shown.

Figure 1

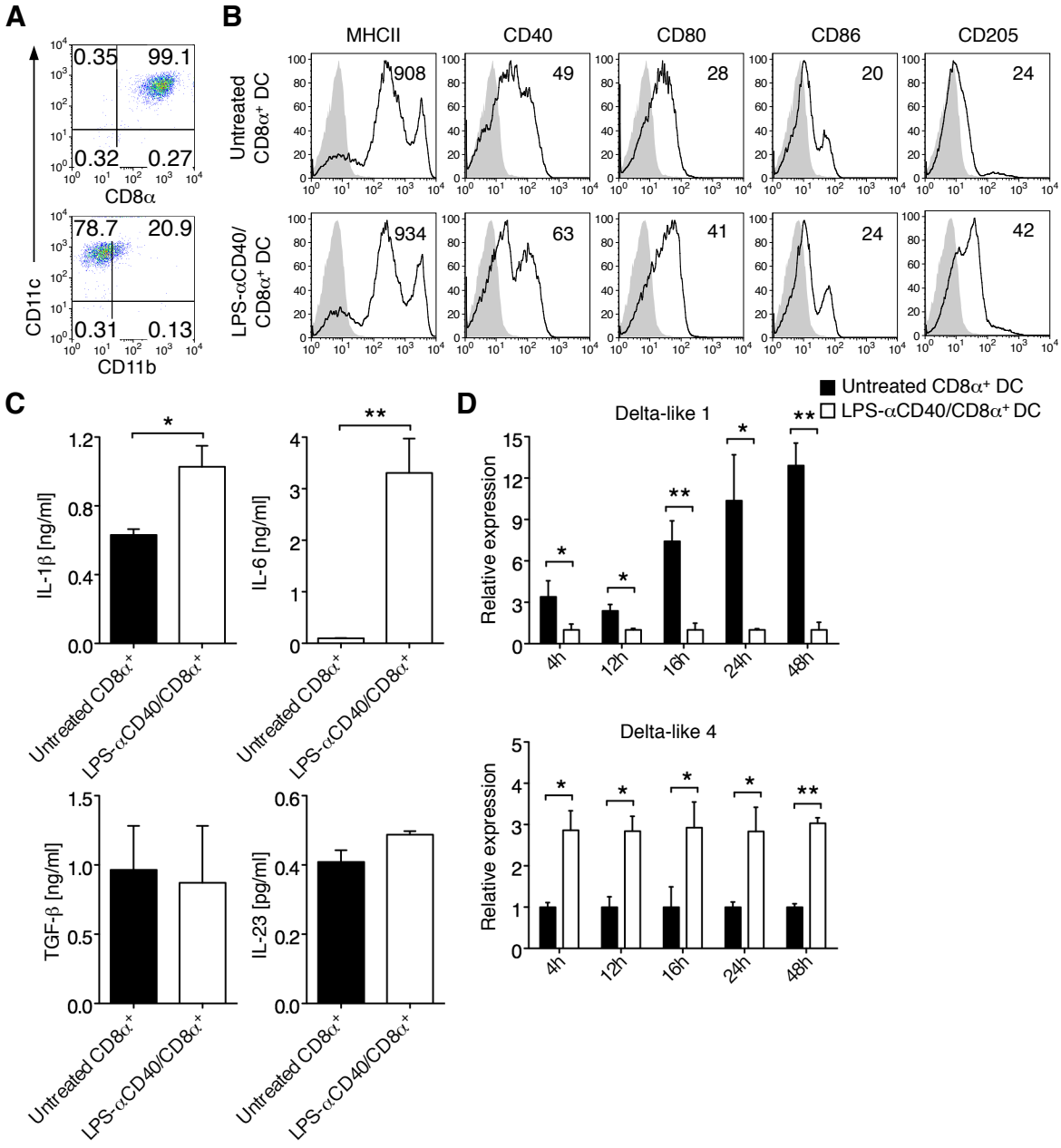

A
0.35
99.1
0.32
0.27
CD8α
78.7
20.9
0.31
0.13
CD11c
CD11b
B
MHCII
CD40
CD80
CD86
CD205
Untreated CD8α+ DC
LPS-αCD40/ CD8α+ DC
908
49
28
20
24
934
63
41
24
42
C
IL-1β [ng/ml]
IL-6 [ng/ml]
TGF-β [ng/ml]
IL-23 [pg/ml]
Untreated CD8α+
LPS-αCD40/CD8α+
D
Untreated CD8α+ DC
LPS-αCD40/CD8α+ DC
Delta-like 1
Delta-like 4
Relative expression
4h
12h
16h
24h
48h

Figure 2

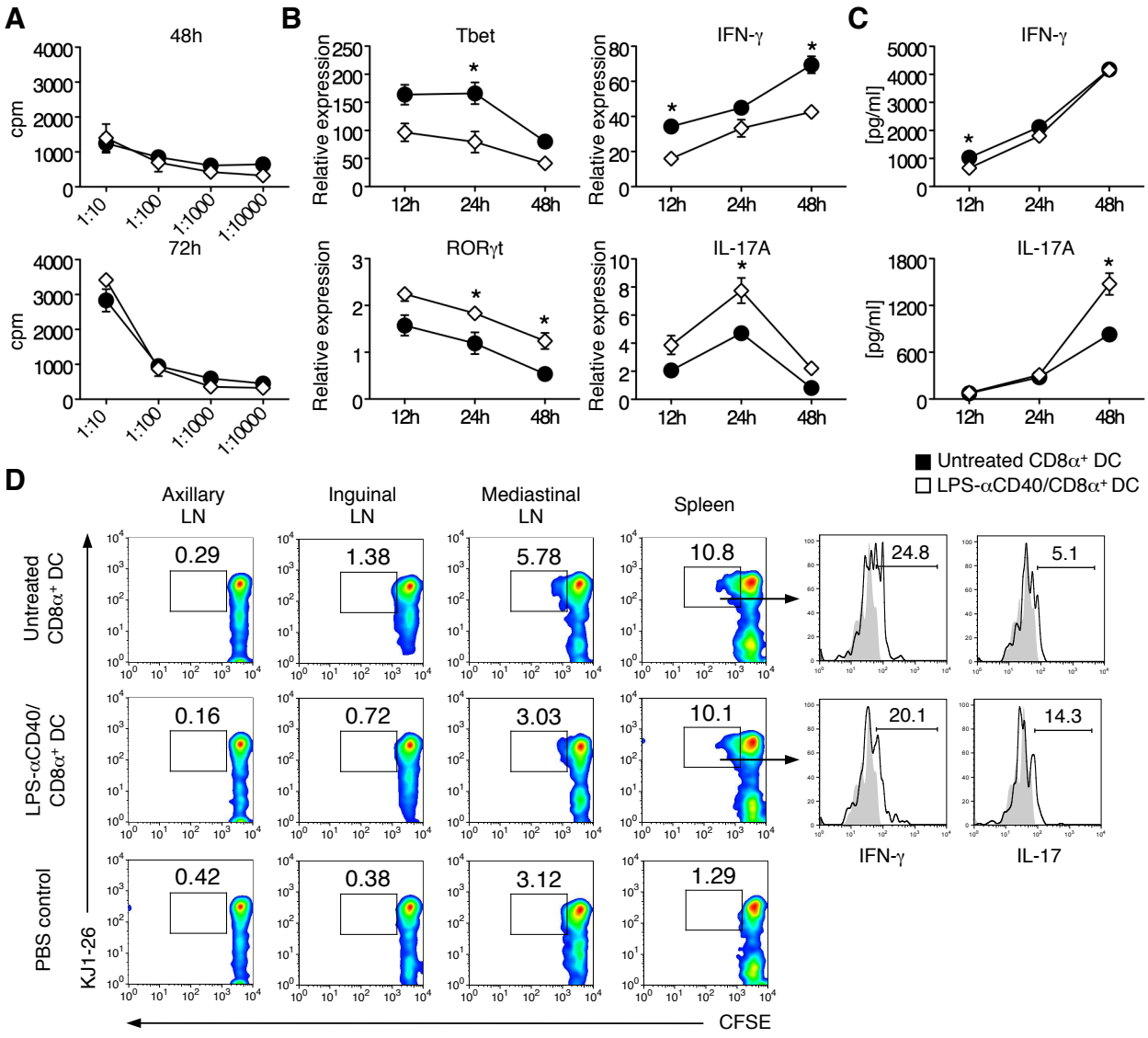

A
48h
cpm
1:10
1:100
1:1000
1:10000
72h
B
Tbet
IFN-γ
RORγt
IL-17A
Relative expression
12h
24h
48h
C
IFN-γ
IL-17A
[pg/ml]
Untreated CD8α+ DC
LPS-αCD40/CD8α+ DC
D
Axillary LN
Inguinal LN
Mediastinal LN
Spleen
Untreated CD8α+ DC
LPS-αCD40/ CD8α+ DC
PBS control
0.29
1.38
5.78
10.8
24.8
5.1
0.16
0.72
3.03
10.1
20.1
14.3
0.42
0.38
3.12
1.29
IFN-γ
IL-17
KJ1-26
CFSE

Figure 3

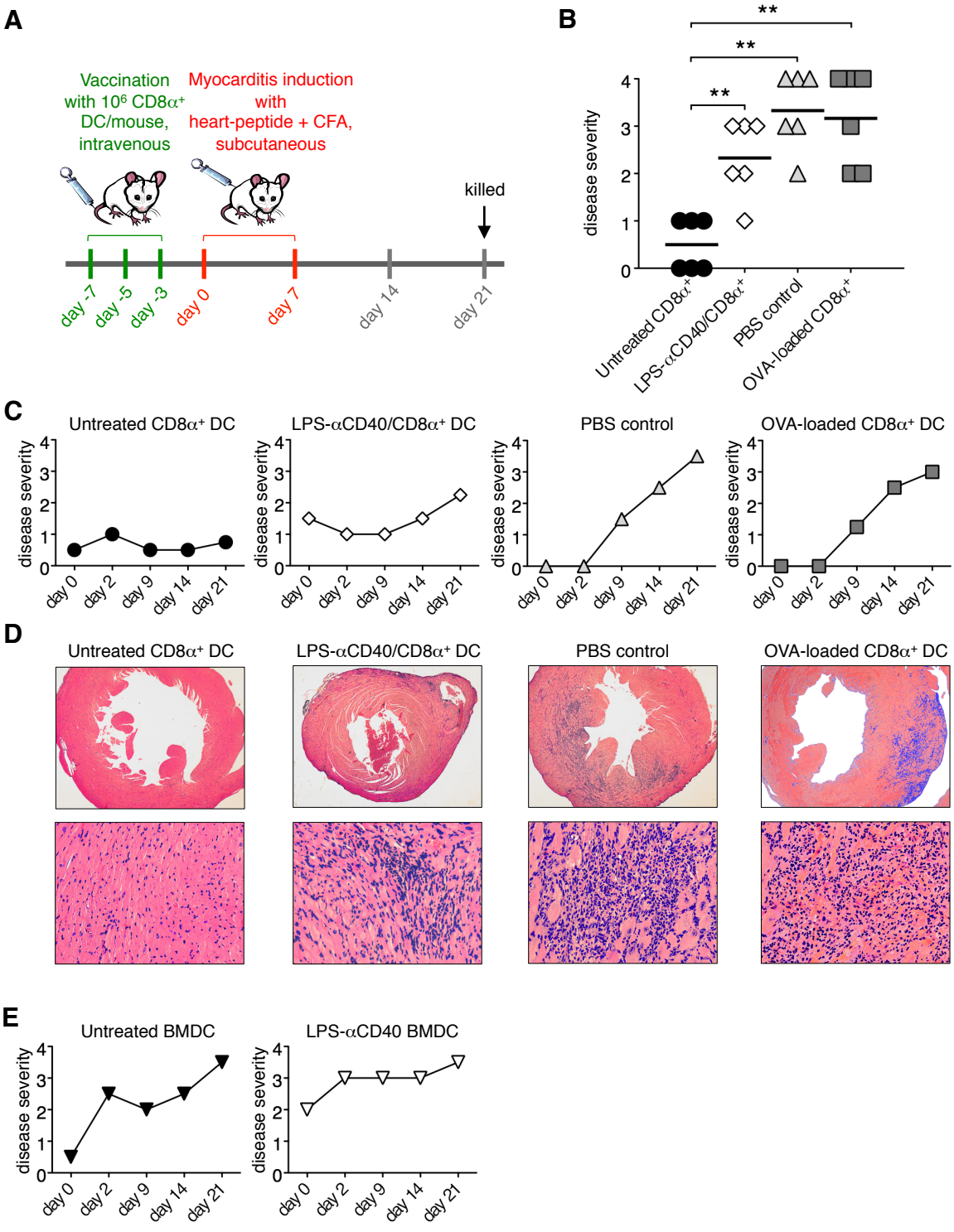

A
Vaccination with 10⁶ CD8α⁺ DC/mouse, intravenous
Myocarditis induction with heart-peptide + CFA, subcutaneous
killed
day -7
day -5
day -3
day 0
day 7
day 14
day 21
B
**
**
**
disease severity
Untreated CD8α⁺
LPS-αCD40/CD8α⁺
PBS control
OVA-loaded CD8α⁺
C
Untreated CD8α⁺ DC
LPS-αCD40/CD8α⁺ DC
PBS control
OVA-loaded CD8α⁺ DC
disease severity
day 0
day 2
day 9
day 14
day 21
D
Untreated CD8α⁺ DC
LPS-αCD40/CD8α⁺ DC
PBS control
OVA-loaded CD8α⁺ DC
E
Untreated BMDC
LPS-αCD40 BMDC
disease severity
day 0
day 2
day 9
day 14
day 21

Figure 4

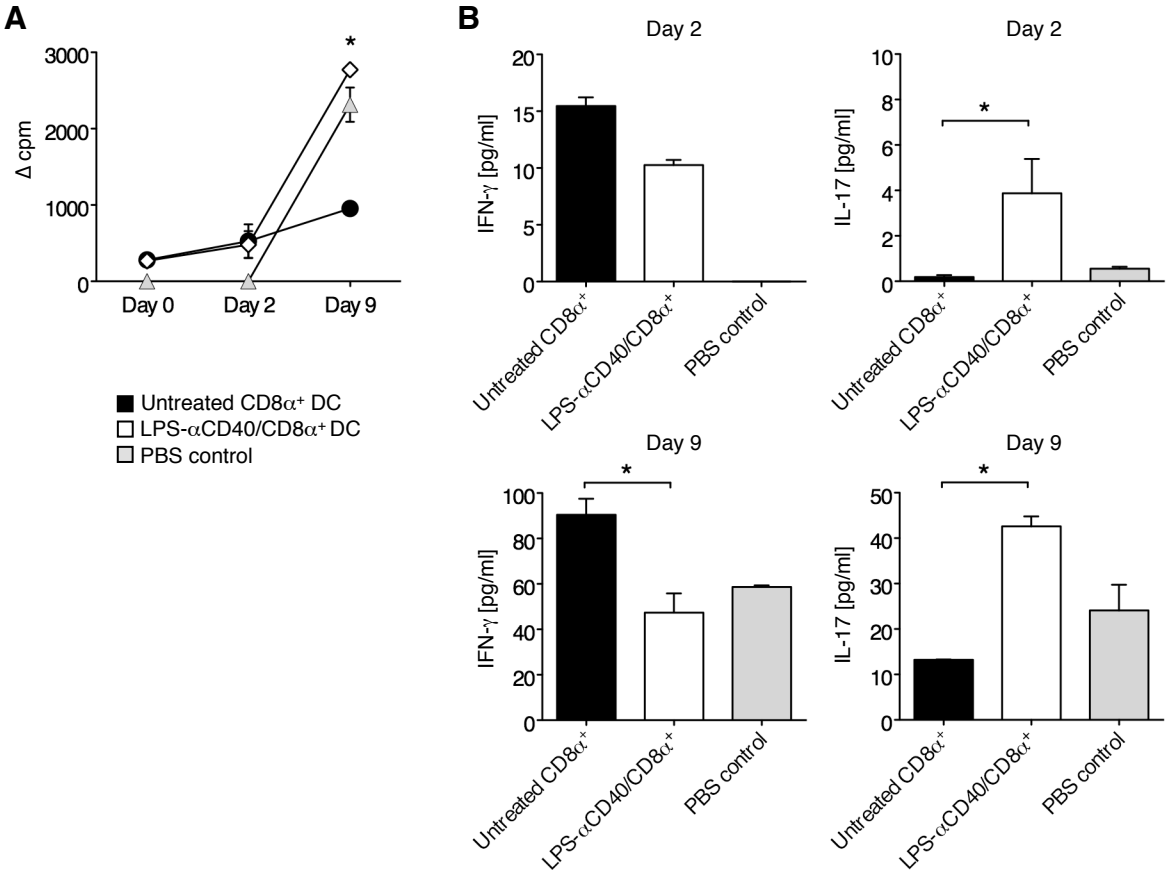

A
3000
2000
1000
0
Δ cpm
*
Day 0
Day 2
Day 9
Untreated CD8α+ DC
LPS-αCD40/CD8α+ DC
PBS control
B
Day 2
IFN-γ [pg/ml]
20
15
10
5
0
Untreated CD8α+
LPS-αCD40/CD8α+
PBS control
Day 2
IL-17 [pg/ml]
10
8
6
4
2
0
*
Untreated CD8α+
LPS-αCD40/CD8α+
PBS control
Day 9
IFN-γ [pg/ml]
100
80
60
40
20
0
*
Untreated CD8α+
LPS-αCD40/CD8α+
PBS control
Day 9
IL-17 [pg/ml]
50
40
30
20
10
0
*
Untreated CD8α+
LPS-αCD40/CD8α+
PBS control

Figure 5

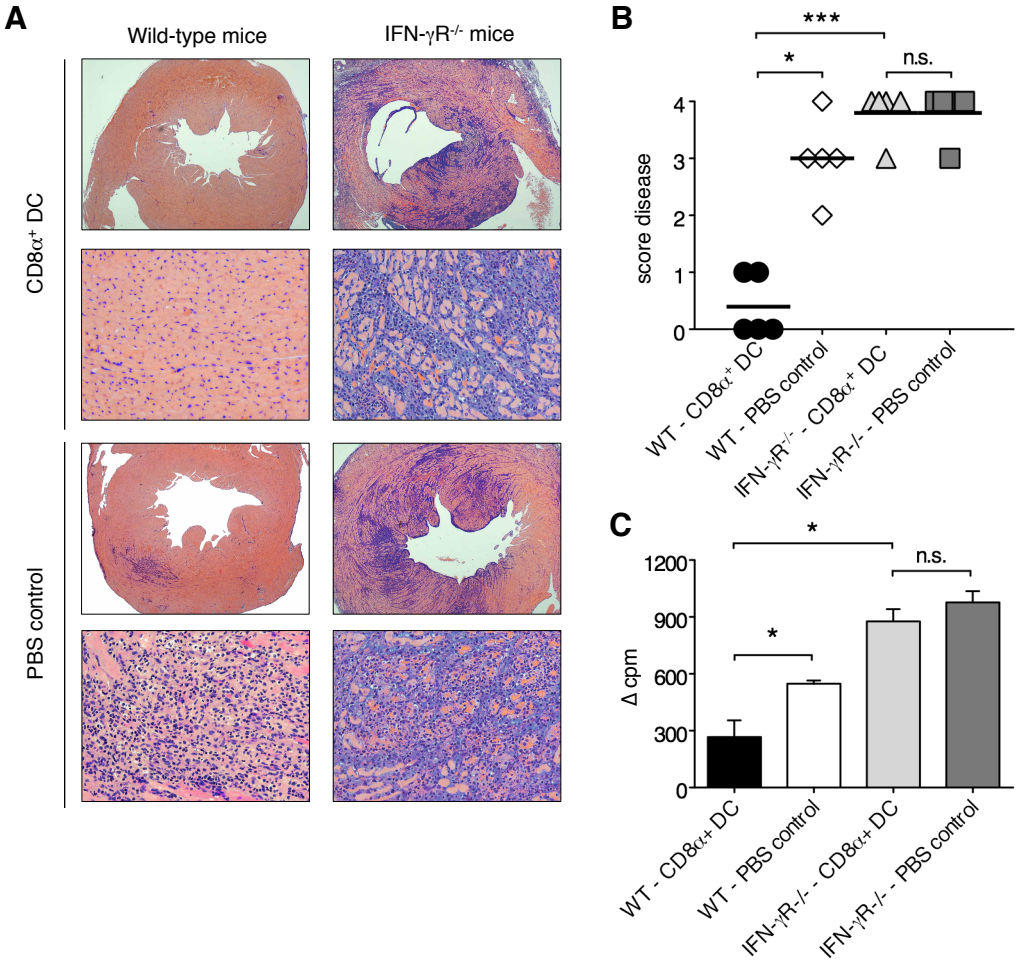
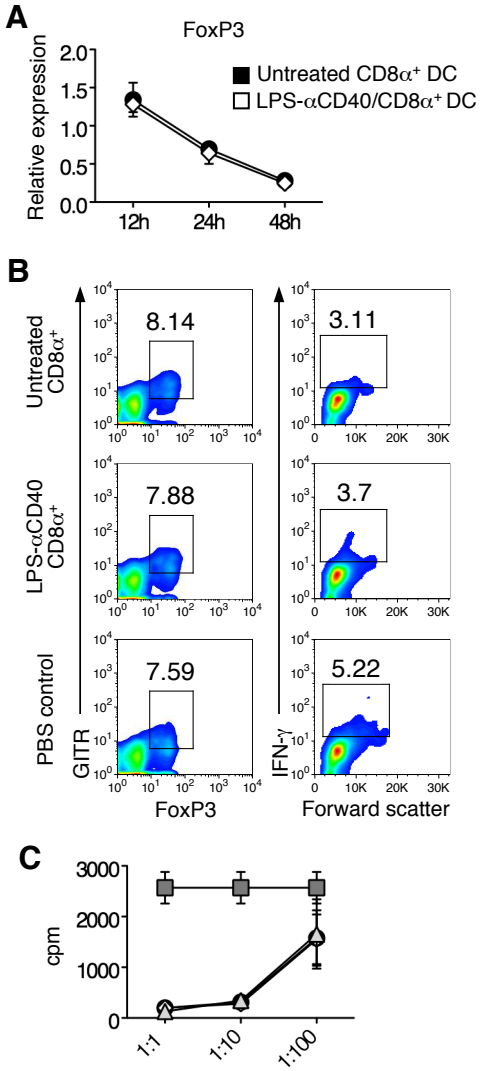

A
Wild-type mice
IFN-γR-/- mice
CD8α+ DC
PBS control
B
***
*
n.s.
score disease
0
1
2
3
4
WT - CD8α+ DC
WT - PBS control
IFN-γR-/- - CD8α+ DC
IFN-γR-/- - PBS control
C
*
n.s.
*
Δ cpm
0
300
600
900
1200
WT - CD8α+ DC
WT - PBS control
IFN-γR-/- - CD8α+ DC
IFN-γR-/- - PBS control

Figure 6


A
FoxP3
Untreated CD8α+ DC
LPS-αCD40/CD8α+ DC
Relative expression
2.0
1.5
1.0
0.5
0.0
12h
24h
48h
B
Untreated CD8α+
LPS-αCD40 CD8α+
PBS control
GITR
FoxP3
8.14
7.88
7.59
IFN-γ
Forward scatter
3.11
3.7
5.22
0
10K
20K
30K
C
cpm
3000
2000
1000
0
1:1
1:10
1:100